Detection of H5N1 High-Pathogenicity Avian Influenza Virus in Meat and Tracheal Samples from Experimentally Infected Chickens

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SUMMARY. The Asian H5N1 highly pathogenic avian influenza (HPAI) virus causes a systemic disease with high mortality of poultry and is potentially zoonotic. In both chickens and ducks, the virus has been demonstrated to replicate in both cardiac and skeletal muscle cells. Experimentally, H5N1 HPAI virus has been transmitted to chickens through the consumption of raw infected meat. In this study, we investigated virus replication in cardiac and skeletal muscle and in the trachea of chickens after experimental intranasal inoculation with the H5N1 HPAI virus. The virus was detected in tissues by real-time reverse transcription–polymerase chain reaction (RRT-PCR) and virus isolation, and in the trachea by RRT-PCR and a commercial avian influenza (AI) viral antigen detection test. A modified RNA extraction protocol was developed for rapid detection of the virus in tissues by RRT-PCR. The H5N1 HPAI virus was sporadically detected in meat and the tracheas of infected birds without any clinical sign of disease as early as 6 hr postinfection (PI), and was detected in all samples tested at 24 hr PI and later. No differences in sensitivity were seen between virus isolation and RRT-PCR in meat samples. The AI viral antigen detection test on tracheal swabs was a useful method for identifying infected chickens when they were sick or dead, but was less sensitive in detecting infected birds when they were preclinical. This study provides data indicating that preslaughter tracheal swab testing can identify birds infected with HPAI among the daily mortality and prevent infected flocks from being sent to processing plants. In addition, the modified RNA extraction and RRT-PCR test on meat samples provide a rapid and sensitive method of identifying HPAI virus in illegal contraband or domestic meat samples.

RESUMEN. Detección de virus H5N1 de influenza aviar de alta patogenicidad en carne y muestras traqueales provenientes de pollos infectados experimentalmente.

El virus H5N1 de influenza aviar de alta patogenicidad Asiático causa una enfermedad sistémica con alta mortalidad en aves domésticas y es potencialmente zoonótico. Se ha demostrado que el virus se replica en las células de la musculatura cardíaca y esquelética tanto en pollos como en patos. El virus H5N1 de influenza aviar de alta patogenicidad se ha transmitido experimentalmente a pollos mediante el consumo de carne cruda infectada. En el presente estudio, se investigó la replicación del virus en el músculo esquelético, en el músculo cardíaco y en la tráquea de pollos después de la inoculación experimental por vía intranasal con virus H5N1 de influenza aviar de alta patogenicidad. El virus se detectó en tejidos mediante la prueba de reacción en cadena por la polimerasa-transcriptasa reversa en tiempo real (por sus siglas en Inglés RRT-PCR) y mediante aislamiento viral, mientras en la tráquea se detectó mediante RRT-PCR y una prueba comercial de detección de antígeno viral de influenza aviar. Para la rápida detección del virus en los tejidos mediante la prueba RRT-PCR, se desarrolló un protocolo modificado de extracción de RNA. A partir de seis horas posteriores a la infección, el virus H5N1 de influenza aviar de alta patogenicidad se detectó esporádicamente en carne y tráqueas de aves infectadas sin ningún signo clínico de la enfermedad y luego se detectó en todas las muestras evaluadas a partir de las 24 horas posteriores a la infección. En las muestras de carne no se observaron diferencias en la sensibilidad entre el aislamiento viral y la prueba RRT-PCR. La detección del antígeno viral de influenza aviar en las muestras de tráquea resultó un método útil para identificar los pollos infectados cuando estos estaban enfermos o muertos, pero fue menos sensible en la detección de aves infectadas cuando estas estaban en el estadio preclínico. Este estudio proporciona información que indica que la evaluación e hisopos traqueales antes del sacrificio, puede identificar aves infectadas con virus H5N1 de influenza aviar de alta patogenicidad en la mortalidad diaria y así prevenir el envío de parvadas infectadas a las plantas de procesamiento. Adicionalmente, el método modificado de extracción de RNA y la prueba RRT-PCR para muestras de carne, proporcionan un método rápido y sencillo de identificación del virus H5N1 de influenza aviar de alta patogenicidad en muestras de contrabando ilegal o muestras de carne del mismo país o región.

Key words: avian influenza, high-pathogenicity H5N1, infected chickens, infected tissues and trachea, virus detection, real time RT-PCR, virus isolation, highly pathogenic avian influenza

Abbreviations: AAF = aminoallantoic fluid; AI = avian influenza; BHI = brain–heart infusion; C_t = cycle threshold; EID_{50} = 50% egg-infectious dose; EZ1 = EZ1 viral RNA kit; GI = gastrointestinal; HA = hemagglutinin; HPAI = highly pathogenic avian influenza; IPC = internal positive control; LPAI = low-pathogenicity avian influenza; MDL = minimum detection level; NA = neuraminidase; NVSL = National Veterinary Services Laboratories; PI = postinoculation; QR = RNeasy minikit; QV = viral RNA minikit; RRT-PCR = real-time reverse transcription–polymerase chain reaction; RT-PCR = reverse transcription–polymerase chain reaction; SPF = specific-pathogen-free; VI = virus isolation

Avian influenza (AI) virus has emerged as a serious threat to the poultry industry with the spread of H5N1 highly pathogenic avian influenza (HPAI) virus over three continents. The virus presents an unusual threat because it has the potential to be zoonotic and because this strain of virus has been found in wild birds, which complicates the control of virus spread. Wild aquatic birds including waterfowl and shorebirds are believed to be the natural hosts of all AI viruses, and infection of the natural host with AI virus typically does not produce any disease or clinical illness (17,40,41). Viruses isolated from wild birds, with few exceptions, are of low pathogenicity when tested in chickens, but the current H5N1 HPAI

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virus, which originated in Asia is an exception to this rule. Some strains of this H5N1 HPAI virus lineage can not only infect wild birds but caused outbreaks of severe clinical disease in several waterfowl and wild bird species including swans, geese, and ducks (10,33,42).

AI viruses are classified based on the antigenic diversity of their two surface glycoproteins: hemagglutinin (HA) encoded by segment 4 and neuraminidase (NA) encoded by segment 6 of the viral RNA genome. There are 16 known HA subtypes of AI viruses, represented by H1 through H16, and nine known NA subtypes, represented by N1 through N9 (12,35). Based on the clinical disease associated with AI infections in chickens, the AI viruses have been further classified into two pathotypes, low-pathogenicity AI (LPAI) and HPAI (36). Infection with LPAI virus in chickens is a mucosal infection that experimentally in specific-pathogen-free (SPF) chickens is usually subclinical, but when complicated by other bacterial or viral infections may result in respiratory disease, drops in egg production, and mortality in severe cases (14,20,29). The HPAI virus on the other hand produces both mucosal and systemic infections that by definition cause high mortality in both experimentally and naturally infected chickens. Only the H5 and H7 subtypes of AI viruses have been reported to be highly pathogenic, but most H5 and H7 viruses are of low pathogenicity (6,7,35).

The HPAI viruses, including the H5N1 virus, that are currently circulating in Europe, Asia, and Africa, appear to have an extremely wide host range and have crossed the host species barrier into humans, cats, and other mammals, likely through direct contact with AI virus–infected wild aquatic birds or poultry (1,5,15,21,34,38,39). According to the latest World Health Organization report (as of April 11, 2007; available at http://www.who.int/csr/disease/avian_influenza/country/en/) there were 291 confirmed H5N1 infections with 172 deaths in humans. Although sustained human-to-human transmission of the virus has not been reported, this could occur through genetic changes within the viral genome, enabling the virus to become more human-adapted.

For LPAI infections in chickens, viral replication is primarily restricted to the respiratory and gastrointestinal (GI) tracts, whereas during HPAI infections viral replication not only occurs in the respiratory and GI tracts, but also systemically in the blood, brain, bone marrow, and tissues from heart, spleen, lungs, and skeletal muscle (breast and thigh meat) (16,23,24,26,30). The severity of clinical disease and the spread of the virus in multiple organs were found to be more pronounced in experimentally infected chickens than in other birds, including quail and ducks (23).

HPAI viruses have been isolated from poultry meat and eggs (6,24,27,28,38) and the possible infection of consumers by HPAI viruses from eating improperly cooked food products is a potential concern. However, no human infections of H5N1 have been associated with eating properly cooked poultry products. Experimental evidence of transmission of virus to chickens through consumption of raw infected chicken meat has been reported for some but not all HPAI viruses (35). Field evidence of infection of tigers and leopards by eating raw H5N1 HPAI virus–infected poultry carcasses also suggest a possible role of virus transmission in food to some mammals (18). The HPAI viruses have been shown to replicate in tissues of lung, trachea, liver, spleen, brain, heart, and skeletal muscle from naturally infected chickens, quail, and ducks (2). Eating or contact with raw or undercooked meat or offal from HPAI-infected poultry is a theoretical mechanism for transmission of HPAI virus to humans. In the United States, a system of meat inspection is in place to ensure food safety. In addition, screening of poultry flocks by serologic tests, antigen-capture enzyme-linked

immunosorbent assay tests, or real-time reverse transcription–polymerase chain reaction (RRT-PCR) on oropharyngeal or tracheal swabs are commonly performed in the United States to assure freedom from infection. The addition of a rapid and sensitive diagnostic test for the identification of AI virus in meat could provide an additional tool to guarantee the safety of meat. In this study we monitored the localization and replication of the HPAI virus H5N1 in the trachea and different tissues of experimentally infected chickens. The results show early detection of the virus in infected chickens with no clinical signs of disease and high correlation of test results from four different test methods with specimens sampled from clinically ill chickens.

MATERIALS AND METHODS

Virus inoculum and bird experiment design. All experiments with live viruses were carried out in U.S. Department of Agriculture–certified biosafety level–3 agriculture containment facilities at the Southeast Poultry Research Laboratory. All experiments using animals were conducted under approved protocols of the Institutional Animal Care and Use Committee. The HPAI viruses A/Whooper Swan/Mongolia/244/05 (H5N1) and the LPAI virus A/chicken/AL/7395/75 (H4N8) were propagated in allantoic cavities of 9-to-11-day-old embryonating chickens' eggs following incubation at 37 C. Amnioallantoic fluid (AAF) was harvested 30–48 hr postinoculation (PI) from HPAI-infected eggs and 60–72 hr PI from LPAI-infected eggs and stored in aliquots at −70 C. The 50% egg-infectious dose (EID_{50}) titer of the virus was determined by serial dilutions in embryonating chicken eggs as described (31). In bird experiments 3-to-4-wk-old SPF White Leghorn chickens (*Gallus gallus domesticus*) from the Southeast Poultry Research Laboratory flocks were inoculated intranasally with 10^5 EID_{50}'s in 0.1 ml volume of A/Whooper Swan/Mongolia/244L/05 (H5N1). A total of 96 birds were used in this study including six control birds, which were not inoculated with the virus. Both inoculated and uninoculated (control) birds were housed in self-contained isolation units (Mark 4, Controlled Isolation Systems; San Diego, CA) that were ventilated with high-efficiency particulate-filtered air. Four isolation units were used, one for the six control birds and three for the 90 inoculated birds, each housing 30 birds.

Sample types and collection. Two types of samples were analyzed from experimentally infected birds: tracheal swabs and tissues (heart, breast, and thigh meat). Inoculated birds were sampled every 6 hr at 6, 12, 18, 24, 30, 36, 42 and 48 hr PI whereas the control birds (uninoculated) were sampled only twice, at 0 and 48 hr. At least 10 birds were sampled at each time point except the control birds, for which 3 birds were sampled twice, at 0 and 48 hr. Infected birds were observed for clinical signs prior to sampling. The infected birds were wing-banded and sampled consecutively according to their band number until the birds started showing clinical signs of disease, then the dead and the sick birds were preferentially sampled. Live birds were euthanatized with sodium pentobarbital (100 mg/kg body weight, intravenously) for sampling of tissues. For the tracheal swabs, birds were swabbed from the upper or the lower sections of the trachea, and the samples were alternatively tested by either RRT-PCR or antigen capture test to reduce bias. For RRT-PCR the swabs were suspended in 500 μl of brain–heart infusion media (BHI) and stored at 4 C until processed. For antigen-capture test the swabs were suspended in 250 μl kit-supplied buffer and assayed according to the manufacturer's instructions as described below. All tissue samples were estimated to weigh between 0.5 and 1.0 g based on the size of the sample taken. Tissues of breast, thigh, and heart were sampled in triplicate from each bird at a given time point and numbered as 1, 2, and 3. Replicate 1, used for virus isolation (VI), was collected in 1.5-ml microcentrifuge tubes and stored at −70 C until used. Replicates 2 and 3, used for RNA extraction, were collected in 2-ml collection tubes containing 250 μl BHI, 750 μl Trizol LS (Invitrogen Corp., Carlsbad, CA), and approximately 600 mg of zirconia/silica beads (0.5 mm, BioSpec Products Inc., Bartlesville, OK). Replicate 2 was

briefly stored at 4 C prior to RNA extraction and replicate 3 was stored at −70 C and used later for RNA extraction after one freeze–thaw cycle.

Release of virus from tissue samples for VI. To release the virus from replicate 1, frozen tissues (kept at −70 C) were mixed with sterile sand and macerated manually by grinding with a pestle. The macerated tissues were suspended in BHI to a final concentration of 10% (w/v), vortexed, and then centrifuged at 6000 × g for 5 min. The pellet was discarded and the supernatant was collected for detection of the virus by VI as described below. Tissues from replicates 2 and 3 were macerated with a FastPrep FP 120 tissue disrupter (Savant Instruments Inc., Holbrook, NY) with replicate 2 being processed from fresh tissue and replicate 3 (kept at −70 C) being thawed at room temperature for 15 min prior to maceration. The time of maceration varied depending on tissue types. The heart and thigh meat were macerated on average for 60 sec (3 × 20 sec) and the breast meat was macerated for 40 sec (2 × 20 sec). The samples were individually examined to assure sample maceration was substantially complete. After the maceration was complete, the tissues were subjected to RNA extraction.

Extraction of RNA from tracheal swabs. RNA was extracted from tracheal swabs with QIAmp RNeasy mini kit (Qiagen Inc., Valencia, CA) according to the National Veterinary Services Laboratories (NVSL) standard operating procedure (9). Five hundred microliters of swab material was used per sample for extraction of RNA and the RNA was eluted with 50 μl of RNase-free water.

Modified protocol for extraction of viral RNA from tissues. The modified RNA extraction protocol consisted of two steps. In the first step, macerated tissues were vortexed with Trizol LS and 0.2 ml chloroform and centrifuged at 10,000 × g to separate the upper aqueous phase containing the RNA from the lower organic phase consisting of phenol and chloroform. The aqueous phase, referred to as the Trizol extracts, was collected and used as the sample material for isolation of RNA with the MagMAX^{TM}-96 AI/ND viral RNA isolation kit (Ambion, Austin, TX) using the KingFisher 24 automated nucleic acid purification system from Thermo Electron (Waltham, MA) according to the manufacturer's (Ambion) instructions.

The Trizol extracts were also used as sample material for isolation of RNA by other commercially available RNA extraction kits or methods including Trizol LS (Invitrogen), RNeasy minikit (QR) (Qiagen), viral RNA minikit (QV) (Qiagen), and EZ1 viral RNA kit (EZ1) (Qiagen) according to the manufacturer's instructions, except for Trizol LS and QR, where the RNA extractions were performed according to NVSL protocols (9). The sample materials (Trizol extracts) were diluted appropriately according to the instructions of the individual protocol and the extracted RNA was eluted with 50 μl of RNase-free water or elution buffer, except for EZ1 where the elution volume was 75 μl.

Detection of AI virus in tracheal swabs. The detection of H5N1 virus in tracheal swabs was performed using two methods: RRT-PCR and a commercial type-A influenza antigen-capture immunoassay kit (Flu-Detect®, Synbiotics Inc, San Diego, CA). The RRT-PCR was carried out via amplification of the viral RNA matrix gene using lyophilized reagents as described below. For antigen-capture immuno-assay the swabs were dispensed in the kit-supplied lysis buffer and the liquid expressed from the swab was tested for the presence of type-A influenza antigen according to the manufacturer's instructions.

Detection of the virus in infected tissues by VI. Tissue extracts from replicate 1 were used directly for detection of the virus by VI in 9-to-11-day-old embryonating chicken eggs. The eggs were inoculated with 0.2 ml of tissue homogenates in triplicate (for each sample) and incubated at 37 C for 2–3 days; the AAF was collected from each egg individually. The hemagglutination assay was performed with 0.5% chicken red blood cells in phosphate-buffered saline by standard procedures on AAF (31) to determine if AI virus was present.

RRT-PCR. The RRT-PCR test targeted the amplification of the viral matrix gene and was carried out using lyophilized reagents as previously described (9). This protocol was a modification of the previous protocol (32) in which lyophilized reagents were used replacing the liquid master reaction mixture for RRT-PCR. An internal positive control (IPC) was added to the master mix to help identify false negative results. The IPC shared the same primer binding sites as the target amplicon (viral matrix

Table 1. Comparison of QR and Trizol LS for quality of extraction of RNA from tissues spiked with LPAI virus.

| Tissue/dilution[A] | Cycle threshold value (C_t) | | |
	QR	Trizol LS	C_t difference (QR − Trizol LS)
BHI + virus (undiluted)	22.42	17.97	4.45
10^{-1} Dilution	25.14	20.31	4.83
10^{-2} Dilution	28.33	24.08	4.25
10^{-3} Dilution	32.43	27.48	4.95
Breast + virus (stock)	32.26	0 (Inhibition)	—
10^{-1} Dilution	36.70	29.61	7.09
10^{-2} Dilution	39.82	25.81	14.01
10^{-3} Dilution	0	27.35	—
Thigh + virus (stock)	31.62	0 (Inhibition)	—
10^{-1} Dilution	34.53	36.99	−2.46
10^{-2} Dilution	37.79	27.97	9.82
10^{-3} Dilution	41.25	27.54	13.71
Heart + virus (stock)	34.61	35.55	0.91
10^{-1} Dilution	39.43	25.74	13.69
10^{-2} Dilution	0	26.13	—
10^{-3} Dilution	0	27.49	—

[A]Tissues weighing between 0.5 and 1.0 g wet were suspended in 250 μl BHI spiked with 50 μl of A/chicken/AL/7395/75 (H4N8) (EID$_{50}$ of $10^{8.5}$/ml) plus QR RLT buffer (500 μl) or Trizol LS (750 μl) and macerated with a tissue disruptor as described in Materials and Methods. The control (BHI plus virus only) was subjected to similar treatment as tissues. Tissue extracts (RLT or Trizol LS extracts) were subjected to RNA extraction according to NVSL protocols (9). The RNA was serially diluted with RNase-free water (as shown) and 8 μl RNA from each dilution used per 17 μl of master reaction mixture for RRT-PCR (25 μl reaction volume) using lyophilized reagents with IPC as described in the materials and methods. The presence of RRT-PCR inhibitors in the samples monitored with an IPC as described (9).

gene) but had a unique internal sequence that was detected with an IPC specific probe. During RRT-PCR the amplification of the viral RNA was monitored with a matrix probe in the FAM channel and the amplification of the IPC with the IPC probe in the Texas Red channel. The RRT-PCR test was run for 40 cycles of PCR amplification where cycle threshold (C_t) values of 35 cycles or less were considered positive and C_t values between 35 and 40 were considered suspect and were retested by RT-PCR to confirm the results.

Histopathology and immunohistochemistry. To determine the mechanism of the infection and dissemination of AI virus, tissues (nasal cavity, heart, brain, and spleen) from three chickens at each time point were collected, fixed in 10% neutral-buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Duplicate unstained sections were immunohistochemically stained to demonstrate the presence of influenza A viral protein as described (35). The primary antibody was a monoclonal antibody specific for type A influenza viral nucleoprotein (P13C11). The secondary antibody was a goat anti-mouse IgG tagged with horseradish peroxidase (Southern Biotech, Birmingham, AL) and the chromogen 3,3-diaminobenzidine (DAB plus kit, Zymed, San Fancisco, CA) was used for visualization.

RESULTS

Development and validation of a modified protocol for extraction of AI virus RNA from tissues. We tested two commercial RNA extraction kits, RNeasy minikit QR (Qiagen) and the Trizol LS (Invitrogen), for extraction of viral RNA from breast meat, thigh meat, and heart from SPF chickens with the LPAI virus A/chicken/AL/7395/75 (H4N8) spiked into the sample.

Table 2. Comparison and evaluation of different commercially available RNA extraction kits for isolation of RNA from Trizol LS extracts of different tissues.

Tissue type	Trizol LS extracts[A]	Average cycle threshold (C_t) of RNA				
		QR	QV	Trizol LS	EZ1	MM[B]
Breast	50 µl	28.78	27.92	27.38	28.01	27.14
Thigh	50 µl	28.63	28.88	28.34	28.92	26.87
Heart	50 µl	27.96	28.34	27.20	27.48	25.89

[A]Trizol LS extracts of tissues were prepared as described in the footnote to Table 1. Fifty microliters of Trizol LS extracts were diluted to 500 µl, 140 µl, 250 µl, and 400 µl for extraction with QR, QV, Trizol LS, and EZ1, respectively, according to the instructions for individual protocols.
[B]MagMAX[TM]-96 AI/ND Viral RNA isolation kit (Ambion).

Results of RRT-PCR and comparisons of the C_t values of the RNA extracted from serial dilutions of the virus in BHI indicate poor RNA extractability with QR compared to Trizol LS (Table 1). The RNA extracted from the tissues with both the protocols exhibited higher C_t values compared to BHI controls, indicating presence of RT-PCR inhibitors in the extracted RNA. To check the level of inhibitors in the RNA extracted from tissues, the RNA was serially diluted and then subjected to RRT-PCR in the presence of an IPC. The results (Table 1) show higher C_ts corresponding to the RNA extracted by QR compared to the Trizol LS method at all dilutions except for the undiluted stocks. Based on the C_t values corresponding to the IPC, the levels of RT-PCR inhibitors were found to be similar in the RNA extracted by both the methods (data not shown), suggesting that the higher C_ts with the RNA extracted by QR is likely due to poor RNA extraction efficiency. Comparison of the C_t values of the RNA extracted by Trizol LS from tissues and BHI controls at different dilutions indicates higher C_ts with the RNA extracted from the tissues compared to BHI controls at 0, 10^{-1}, and 10^{-2} dilutions, but similar C_ts at 10^{-3} dilution. These results indicate similar yields of the RNA extracted by Trizol LS from the spiked tissues or spiked BHI controls and that the higher C_ts at 0, 10^{-1}, and 10^{-2} dilutions were due to the presence of inhibitors. Poor yields of RNA with QR compared to the Trizol LS method and strong presence of RT-PCR inhibitors in RNA extracted from tissues with Trizol LS have been previously reported (9).

To investigate whether RT-PCR inhibitors coextracted with RNA from tissues by Trizol LS can be removed by other protocols, the crude tissue Trizol extracts were used as the starting material for isolation of RNA using several commercial RNA extraction kits including QR, QV, EZ1, and MagMAX[TM]-96 (see Materials and Methods). The results (Table 2) show lower C_ts corresponding to the RNA extracted by MagMAX[TM]-96 compared to other protocols, indicating better separation of RT-PCR inhibitors using MagMAX[TM]-96. Therefore, a combination of Trizol LS plus MagMAX[TM]-96 protocol as described above were used for extraction of viral RNA from infected tissues described in this study.

Pathogenicity and detection of the virus in various test samples from chickens experimentally infected with H5N1 A/Whooper Swan/Mongolia/244/05. The pathogenesis of H5N1 in chickens was monitored based on the mortality, morbidity, and detection of the virus in different tissues (breast, thigh, and heart) and tracheal swabs collected from the infected birds at different time points PI. The samples were analyzed for the presence of the virus by RRT-PCR, VI (for tissues only), and commercial antigen-capture immunoassay test (for tracheal swabs only). The results are summarized in Table 3.

The virus was sporadically detected in tissues and trachea of infected birds as early as 6 hr PI, but the birds did not show any clinical signs of infection until 24 hr PI when all infected birds tested positive for AI virus by one or more test methods. The clinical signs of disease in infected birds included depression and dehydration. Gross lesions seen due to infection included enlarged spleens and small thymus. Analysis of breast, thigh, and heart tissues from 30 infected birds (10 at each time point) sampled at 6, 12, and 18 hr PI showed 16 birds positive for AI virus by RRT-PCR and 14 birds positive for AI virus by VI (not shown). The number of birds tested positive at 6, 12 and 18 hr PI were five, five, and six, respectively, by RRT-PCR, and two, two, and 10, respectively, by VI (Table 3). Among the tissues, a total of 90 samples (breast, thigh, or heart) from 30 birds were tested by RRT-PCR and VI, and the number of tissues that tested positive for AI virus were seven breast (23%), 12 thigh (40%), and 10 heart (33%) by RRT-PCR, and seven breast (23%), nine thigh (30%), and 13 heart (43%) by VI. A total of 30 tracheal swabs from the same infected birds sampled between 6 and 18 hr PI (i.e., preclinical) were analyzed; nine birds (30%) tested positive for AI virus by RRT-PCR and only one bird tested positive for AI virus by the antigen-capture test. Almost all of the samples collected from infected birds between 24 and 48 hr PI tested positive for AI virus by all four test methods, indicating close agreement of test results between the test methods for clinically ill or dead birds.

Table 3. Morbidity, mortality, and detection of AI virus at different PI time points in clinical specimens collected from chickens intranasally inoculated with A/Whooper Swan/Mongolia/244L/05 H5N1 HPAI virus.

Time (hr)	Morbidity[A]	Mortality[B]	Detection of AI virus (no. positive/no. examined)[C]							
			Trachea		Breast meat		Thigh meat		Heart	
			RRT-PCR	AI Antigen	RRT-PCR	VI	RRT-PCR	VI	RRT-PCR	VI
0	0/3	0/3	0/3[C]	0/3	0/3	0/3	0/3	0/3	0/3	0/3
6	0/10	0/10	2/10	0/10	0/10	0/10	3/10	1/10	4/10	2/10
12	0/10	0/10	2/10	0/10	3/10	2/10	3/10	1/10	2/10	1/10
18	0/10	0/10	5/10	1/10	4/10	5/10	6/10	7/10	4/10	10/10
24	9/10	0/10	9/10	6/10	9/10	10/10	9/10	10/10	10/10	10/10
30	10/10	0/10	10/10	8/10	10/10	10/10	10/10	10/10	10/10	10/10
36	6/10	4/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
42	—	19/19	19/19	19/19	19/19	19/19	19/19	19/19	19/19	19/19
48	—	11/11	11/11	11/11	11/11	11/11	11/11	11/11	11/11	11/11

[A]The number of sick per total number of birds sampled at each PI time point.
[B]The number of dead birds per total number of birds sampled at each PI time point.
[C]The number of positive samples per the total number of samples tested. All control chickens were negative for AI virus by all test methods and are not included in these numbers.
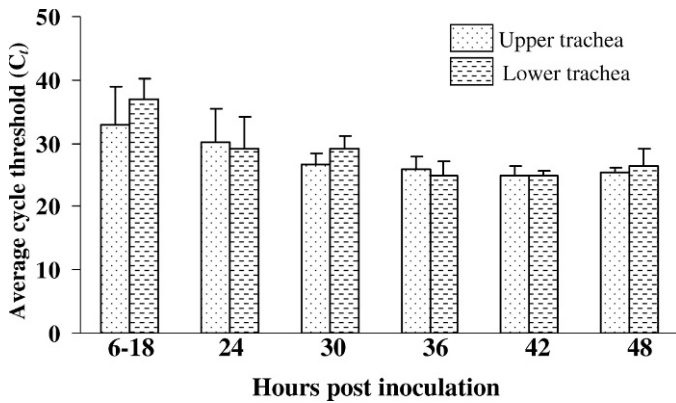
Fig. 1.   Relative distributions of H5N1 in upper and lower trachea of infected chickens at different PI time points. The C_t values corresponding to the RNA extracted from the upper tracheal swabs and the lower tracheal swabs were averaged and plotted against the corresponding time of sampling (PI) as shown. The C_t values corresponding to the swabs sampled between 6 and 18 hr PI were combined, averaged and plotted against 6-18 h PI in the figure. The SDs are shown as error bars. RNA was extracted from swabs with the QR and assayed for RRT-PCR with AI matrix beads plus IPC as described in Materials and Methods.

The tracheal swabs from 34 dead birds and 25 clinically ill birds were tested by RRT-PCR and antigen-capture tests. The number of dead and clinically ill birds that tested positive for AIV were 34/34 and 25/25, respectively, by RRT-PCR, and 35/35 and 20/25, respectively, by antigen-capture test. The infected birds started to die at 36 hr PI and all birds were dead by 48 hr PI. Because of the age and size of the birds, tracheal swabs could not be collected from the same location for use in both antigen-capture and RRT-PCR from each bird. To reduce bias, swabs were alternatively collected from the upper and lower trachea for use in the tests. No apparent difference was seen by sampling of the upper *vs.* lower trachea. The C_ts corresponding to the virus in tissues or trachea decreased at the later PI time points, indicating higher viral concentrations at those sampling times (Fig. 1).

Determination of virus titer in tissues. The virus titers in the infected tissues were determined to compare the sensitivity of detection of the virus between VI and RRT-PCR. A total of 20 VI-positive tissues (heart, breast, and thigh) sampled between 6 and 30 hr PI were subjected to EID_{50} assay to determine the virus titer. The average virus titers ($\log_{10} EID_{50}/g$) of the tissues sampled from infected birds at 6, 12, 18, 24, and 30 hr PI were determined to be 1.96 ± 0.11 (three samples), 2.45 ± 0.07 (two samples), 3.05 ± 1.27 (six samples), 6.09 ± 1.52 (seven samples), and 4.85 ± 0.92 (two samples), respectively (Table 4), indicating a one-log difference in the virus titer between 6 and 18 hr PI while a 3-log difference in the virus titer between 18 and 24 hr PI. The virus titers did not appreciably change between 24 and 30 hr PI, indicating near-saturation of virus replication after 24 hr PI. The above results correlate well with the pathogenicity of the virus because the clinical signs of the H5N1 infection were apparent only after 24 hr PI but not between 6 and 18 hr PI, indicating rapid multiplication of the virus in target tissues between 18 and 24 hr PI. Based on the virus titers and the C_ts corresponding to the virus in the infected tissues (Table 4), a virus titer ($\log_{10} EID_{50}/g$) of 2.2 or higher was found to be detectable by RRT-PCR, suggesting that a virus titer of 2.2 could be the minimum detection level (MDL) of the virus in tissues by RRT-PCR. However, the above MDL is subject to marginal variation based on several factors including the level of inhibitors in

Table 4.   Mean virus titers in tissues sampled from infected chickens at different PI time points.

| | | | Cycle threshold (C_t)[B] | |
Tissue type	Hr PI	$\log_{10} EID_{50}/g$ tissue[A]	Fresh tissue	Freeze–thawed tissue
Heart	6	1.9	0	0
Heart	6	2.1	0	0
Thigh	6	1.9	32.09	0
Heart	12	2.4	37.44	0
Thigh	12	2.5	36.80	0
Breast	18	3.0	33.08	32.71
Breast	18	5.5	28.22	28.30
Heart	18	2.2	39.65	24.25
Heart	18	2.2	40.00	0
Heart	18	2.3	37.49	36.11
Heart	18	3.1	24.05	0
Breast	24	4.0	34.51	36.44
Thigh	24	4.1	38.58	32.15
Thigh	24	5.7	28.12	27.66
Thigh	24	6.6	24.96	24.30
Thigh	24	7.4	27.59	24.60
Thigh	24	7.5	30.28	26.30
Heart	24	7.3	21.82	23.04
Breast	30	4.2	31.12	32.03
Breast	30	5.5	28.51	30.59

[A]Tissue suspensions used for VI were used for EID_{50} assay according to the standard methods (31).
[B]The RNA extractions from fresh and frozen–thawed tissues were as described in the Materials and Methods.

the test samples, the efficiency of RNA extraction, and the quality of the RNA used in RRT-PCR. The above MDL of the virus determined by RRT-PCR was not consistent with the RNA extracted from tissues after freeze–thaw, indicating some inconsistency in the latter procedure. The average virus titers ($\log_{10} EID_{50}/g$) for the tissues sampled at 24 and 30 hr PI were determined to be 6.26 ± 1.40 for thighs (five samples) and 4.56 ± 0.81 for breasts (three samples), indicating some preference for viral replications in the breasts than the thighs in the early clinical stages of infection.

Distribution of H5N1 in breast, thigh, and heart of infected chickens. The distribution of H5N1 HPAI virus in breast, thigh, and heart of infected chickens was monitored by RRT-PCR. Two replicates were sampled from each tissue type at each time point PI, one extracted directly with Trizol LS and the other extracted with Trizol LS after one cycle of freeze–thaw as described in Materials and Methods. The freeze–thaw cycle was included as an alternative protocol which could have additional effect on the release of the virus from infected tissues or it could simulate long-term storage of the samples. As in tracheal swabs, the detection of H5N1 HPAI virus in tissues from infected birds was sporadic between 6 and 18 hr PI (Table 3). The average C_ts corresponding to the viral RNA extracted from tissues sampled between 6 and 18 hr PI were between 34 and 36 whereas the C_ts corresponding to the viral RNA extracted from tissues sampled between 24 and 48 hr PI were between 24 and 30 (not shown), indicating less virus in tissues in the early stages of infection (between 6 and 18 hr PI) than in the latter stages of infection (between 24 and 48 hr PI). Addition of a freeze–thaw cycle prior to RNA extraction had little effect on the number of positive samples (Figs. 2, 3), but some differences were observed in the sensitivity of the detection of the virus based on the C_t values. The average C_t differences between the RNA extracted from fresh tissues and the RNA extracted from the replicate tissues after one cycle of freeze–thaw [C_t(fresh) − C_t(freeze–thaw)] for the samples collected
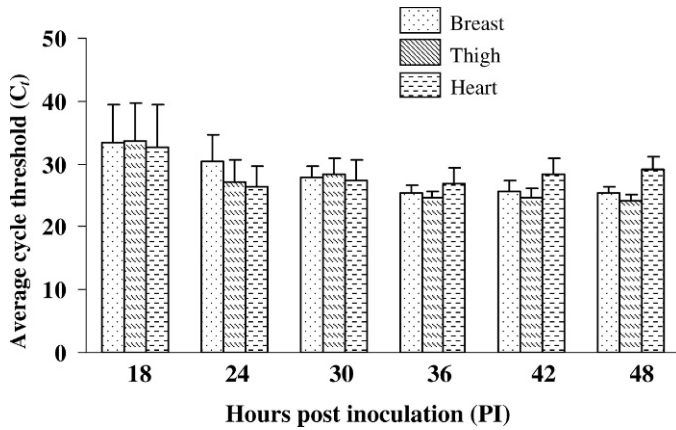
Fig. 2.   Relative distributions of H5N1 in breast, thigh, and heart of infected chickens at different PI time points based on the results of RRT-PCR with viral RNA extracted from tissues of infected chickens after one cycle of freeze–thaw. The C_t values corresponding to the RNA extracted from each tissue type (breast, thigh, or heart) were averaged and plotted against the corresponding time of sampling (PI) as shown. The SDs are shown as error bars. The C_t values corresponding to each tissue type (breast, thigh, or heart) sampled between 6 and 18 hr PI were combined, averaged, and plotted against 6–18-hr PI in the figure. RRT-PCR was carried out with AI matrix beads plus IPC as described in Materials and Methods.
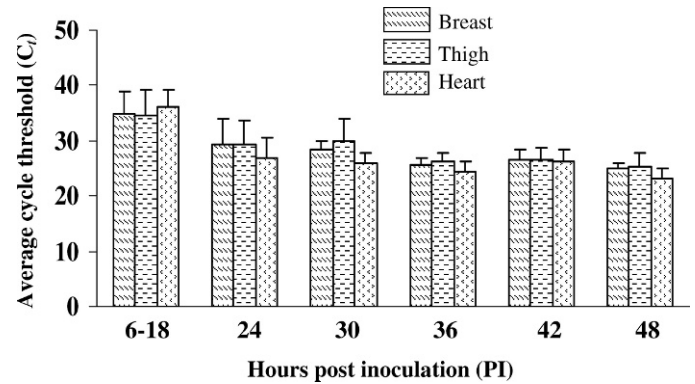

Fig. 3.   Relative distributions of H5N1 in breast, thigh, and heart of infected chickens at different PI time points based on the results of RRT-PCR with viral RNA extracted from fresh (nonfrozen) tissues from infected birds. The C_t values corresponding to the RNA extracted from each tissue type (breast, thigh, or heart) were averaged and plotted against the corresponding time of sampling (PI) as shown. The SDs are shown as error bars. The C_t values corresponding to each tissue type (breast, thigh, or heart) sampled between 6 and 18 hr PI were combined, averaged, and plotted against 6–18-hr PI in the figure. RRT-PCR was carried out with AI matrix beads plus IPC as described in materials and methods.

between 24 and 48 hr PI were 0.15 ($\pm$0.71), 1.83 ($\pm$0.37), and $-$1.68 ($\pm$1.65) for breast, thigh, and heart, respectively, indicating no clear advantage with either method.

Comparison of the C_t values corresponding to the RNA extracted from tissues at different stages of infections show a gradual increase in the level of the virus from the early stages of infection to the latter stages of infection (Figs. 2, 3). Analysis of the relative distributions of the virus between different tissue types (Figs. 2, 3) indicate negligible differences in the content of the virus between breast and thigh but generally lower C_t values in the heart.

Virus dissemination in infected birds. The initial route of virus dissemination in systemic infections is not known. Prior studies in mice suggested a retro-neural dissemination via cranial nerves was responsible for infection in the brain. In this study, AI viral antigen was first demonstrated at 18 hr PI in nasal and sinus epithelium of the middle nasal cavity with associated epithelial necrosis and extension into the underlying lamina propria with AI viral antigen in macrophages of the lamina propria and a few blood capillary endothelia (Table 5). In one bird, AI viral antigen was visualized in occasional splenic macrophages within the periarteriolar lymphatic sheaths and in the associated blood vessel endothelial cells. By 24 hr PI, the AI viral antigen was present in multiple tissues and cell types, most commonly in blood vessel endothelium, cardiac myocytes, neurons, periocular skeletal muscle, and the nasal cavity (Table 5). A similar distribution was seen at later points except that nasal epithelial localization was uncommon at latter time points and osteoblastic infection was seen only at 36–48 hr PI. The intensity of staining was strongest in tissue from dead birds, which corresponds with the highest antigen content.

DISCUSSION

HPAI is a systemic disease in chickens, turkeys, and many other gallinaceous birds. The Asian H5N1 HPAI virus replicates in various tissues, including cardiac muscle and skeletal muscles of infected chickens (2,30), but during the early stages of infection only few

clinical signs of illness are observed, which makes it difficult to distinguish infected birds from healthy birds in chicken flocks. Preclinical H5N1-infected birds processed for human consumption could be a potential source of viral infection and the spread of the disease in humans. This study was designed to evaluate how soon after infection the virus could be detected in chickens. We chose to challenge the chickens with the A/Whooper Swan/Mongolia/244L/2005 H5N1 isolate because it is a representative of the H5N1 HPAI viruses from Asia, Europe, and Africa and has been characterized in several different animal species. The viral challenge was given by a mucosal route of inoculation to mimic a natural exposure and at a modest dose of 10^5 EID$_{50}$, which based on previous experience with Asian H5N1 HPAI lineage viruses should provide a consistent infection of all the birds. Most if not all of the viruses of the Asian H5N1 HPAI lineage are extremely virulent in chickens, and systemic infection with replication in different tissues is expected, and no data are available to suggest that different doses of these viruses will alter the tropism or pathogenesis of the virus. However, the results observed in chickens in this study should be extrapolated with caution for other species because of the well-described differences in pathogenesis of avian influenza between species (30).

Results of RRT-PCR and immunohistochemistry of tissues from chickens infected with high-pathogenicity H5N1 virus indicated sporadic detection of the virus between 6 and 18 hr PI. Results of virus titration on the tissues sampled from infected birds between 6 and 30 hr PI indicated a gradual increase in the virus titers in all tissue types (breast, heart, and thigh) from 6 to 18 hr and then a rapid increase in viral titer from 18 to 24 hr.

Infected birds showed no clinical sign of illness between 6 and 18 hr PI (Table 3). In contrast all infected birds between 24 and 48 hr PI had evidence of clinical disease or death, and the virus was detected in both tissues and tracheal swabs. Direct correlation between the clinical illness and viral replication in multiple organs such as brain, heart, trachea, and cloaca of experimentally infected birds, including chickens, have been reported (3,4,23). The current study shows that the H5N1 was detectable in trachea and tissues of some infected birds within 6 hr PI and in all birds within 24 hr PI. Rapid progression of infection in multiple organs with high-

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Table 5. Localization of AI viral antigen by immunohistochemical staining of nasal cavity, spleen, heart, and brain of chickens from 0 to 48 hr following intranasal inoculation with 10^5 EID_{50} of A/Whooper Swan/Mongolia/244L/2005 (H5N1) HPAI virus.

Sample period (hr)	Tissue site (no. positive/total tested)				Location of AI viral antigen
	Nasal cavity	Spleen	Heart	Brain	
0	0/3[A]	0/3	0/3	0/3	—
6	0/3	0/3	0/3	0/3	—
12	0/3	0/3	0/3	0/3	—
18	2/3 (1.7)[B]	1/3 (1)	0/3	0/3	Nasal epithelium and macrophages in submucosa, splenic blood vessel endothelium and phagocytic cells
24	3/3 (3)	3/3 (4)	3/3 (3.3)	3/3 (2.7)	BVE, CM, N, POM, nasal epithelium and macrophages in submucosa[C]
30	3/3 (2.7)	3/3 (3.7)	3/3 (3)	3/3 (2)	BVE, CM, N, POM, sinus epithelium and macrophages in submucosa
36	3/3 (4)	3/3 (4)	3/3 (4)	3/3 (4)	BVE, CM, N, POM, macrophages in nasal submucosa, osteoblasts
42	3/3 (4)	3/3 (4)	3/3 (4)	3/3 (4)	BVE, CM, N, POM, macrophages in nasal submucosa, osteoblasts
48	3/3 (4)	3/3 (4)	3/3 (4)	3/3 (4)	BVE, CM, N, POM, macrophages in nasal submucosa, osteoblasts

[A]Number with AI viral antigen detected/number tested.
[B]Mean antigen staining score: 1 = rare, 2 = infrequent, 3 = common, 4 = abundant.
[C]BVE = blood vessel endothelium; CM = cardiac myocytes; N = neurons; POM = periocular muscle.

pathogenicity H5N1 virus have been reported in chickens (16,21,34) and other experimental birds and animals including ducks, quail, mice, and ferrets (3,7,23,26). Similar observations have also been reported for chickens infected with other HPAI viruses including H7 (16,35), suggesting a common mode of pathogenesis for HPAI viruses.

For all tissue types the results of RRT-PCR correlate well with VI except for tissues sampled between 6 and 18 hr PI, where fewer samples tested positive for AIV by RRT-PCR than by VI (Table 3). Results of virus titration showed low virus titers (between 1.9 and 2.5 $\log_{10}$ EID_{50}/g) for most tissue types sampled between 6 and 18 hr PI. Fewer AI-positive tissues by RRT-PCR in preclinical infected birds (6–18 hr PI) could be attributed to poor recovery of RNA due to low virus titers, uneven distribution of the virus between the tissues, or the presence of RT-PCR inhibitors in the tissues.

The virus was consistently detected by all methods, including immunohistochemistry, between 24 and 48 hr PI and the results of all test methods correlate well when the birds were either clinically ill or dead. There were some discrepancies in test results between the RRT-PCR and the antigen-capture test for tracheal swabs collected between 6 and 18 hr PI, with the antigen-capture tests identifying few positive samples, which is not unexpected because the available antigen-capture tests are less sensitive than RRT-PCR or VI (8,11). For preclinical infected birds, the RRT-PCR test is needed for its greater sensitivity, but for birds that are sick or dead from HPAI virus, either RRT-PCR or antigen-capture tests on tracheal samples provide comparable results. However, the antigen-capture test is a type-A specific test only, and the subtype cannot be determined as it can be with RRT-PCR. If antigen detection is conducted on tracheas from HPAIV-infected sick or dead birds, the sensitivity is likely sufficient to detect AI infections in flocks and would prevent movement of such flocks to processing.

This study also looked at the distribution of the virus in the infected birds between three different tissues types: breast, thigh, and heart muscle. There appeared to be no major difference in detection of the virus from any of the tissues examined with this particular strain of H5N1 virus. However, not all HPAI viruses have the same tissue tropism. For practical reasons, it would be preferable to use just one tissue for testing if there were concerns about food safety. Because all three sample types gave similar results, the use of heart muscle would be preferred since it is a lower-value product that can be easily obtained from the poultry processing plant.

The testing of the meat samples by RRT-PCR was compared with VI in eggs using a mortar and pestle method to release the virus from the samples. Although this technique is a standard protocol, it is labor intensive and time consuming, which would greatly limit the number of samples that can be processed by a laboratory each day. The RRT-PCR is a rapid test method that allows processing of large number of samples. The tissue disruptor used in this study provided rapid sample processing for RNA extraction from tissues for RRT-PCR. Previous studies have shown that tissues may contain RT-PCR inhibitors or have inefficient RNA extraction, which can result in false negative results (9,19,22,25). Here we compared the efficacy of two commonly used RNA extraction kits, the QR and the Trizol LS reagent, for extraction of RNA from tissues. The Trizol method was more efficient than QR for RNA extraction, but the extracted RNA contained RT-PCR inhibitors that could interfere with RRT-PCR. In the modified protocol used in this study, the RNA was extracted by Trizol LS but purified with magnetic beads using a robotic nucleic acid purification system. The purified RNA had lower levels of RT-PCR inhibitors than the RNA extracted by Trizol LS (Table 2), which helped to detect the virus by RRT-PCR. An internal positive control was used in RRT-PCR to evaluate if RT-PCR inhibitors were present.

The RNA was extracted from tissues by Trizol LS either directly from fresh samples or after one freeze–thaw cycle. The use of a freeze–thaw cycle is a common practice that facilitates ultrastructural damage to the cells and release of intracellular contents (13,37). In this comparison the RNA extraction from fresh tissues gave lower C_t values, but both methods provided similar overall results. This suggests that samples can be frozen if necessary before being processed at the laboratory.

In this study, we demonstrated the utility of targeting clinically ill and dead birds for AI virus testing in tracheal samples by rapid, inexpensive commercial antigen immunoassay kits as a preslaughter testing. In addition, the simplified bench-validated RRT-PCR protocol described in this study can also be used for the detection of AI virus in illegally transported poultry products from H5N1 HPAI–infected countries confiscated at the United States border or in chicken meat from domestic poultry processing plants.

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